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• *H pylori* •

Construction of prokaryotic expression system of 2 148-bp fragment from *cagA* gene and detection of *cagA* gene, CagA protein in *Helicobacter pylori* isolates and its antibody in sera of patients

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Abstract

AIM: To construct a prokaryotic expression system of a *Helicobacter pylori* (*H pylori*) *cagA* gene fragment and establish enzyme-linked immunosorbent assays (ELISA) for detecting CagA and its antibody, so as to understand the manner in which the infection of CagA-expressing *H pylori* (CagA$^+$ *H pylori*) isolates cause diseases.

METHODS: *H pylori* strains in gastric biopsy specimens from 156 patients with positive results in rapid urease test were isolated. PCR was used to detect the frequency of *cagA* gene in the 109 *H pylori* isolates and to amplify a 2 148-bp fragment (*cagA*1) of *cagA* gene from a clinical strain Y06. A prokaryotic expression system of *cagA*1 gene was constructed, and the expression of the target recombinant protein (rCagA1) was examined by SDS-PAGE. Western blotting and immunodiffusion assay were employed to determine the immunoreactivity and antigenicity of rCagA1, respectively. Two ELISAs were established to detect CagA expression in 109 *H pylori* isolates and the presence of CagA antibody in the corresponding patients' sera, and the correlations between infection with CagA$^+$ *H pylori* and gastritis as well as peptic ulcer were analyzed.

RESULTS: Of all the clinical specimens obtained, 80.8% (126/156) were found to have *H pylori* isolates and 97.2% of the isolates (106/109) were positive for *cagA* gene. In comparison with the reported data, the cloned *cagA*1 fragment possessed 94.83% and 93.30% homologies with the nucleotide and putative amino acid sequences, respectively. The output of rCagA1 produced by the constructed recombinant prokaryotic expression system was approximately 30% of the total bacterial protein. rCagA1 was able to bind to the commercial antibody against the whole-cells of *H pylori* and to induce the immunized rabbits to produce antibody with an immunodiffusion titer of 1:4. A proportion as high as 92.6% of the *H pylori* isolates (101/109) expressed CagA and 88.1% of the patients' serum samples (96/109) were CagA antibody-positive. The percentage of CagA$^+$ *H pylori* strains (97.9%) isolated from the biopsy specimens of peptic ulcer appeared to be higher than that from gastritis (88.5%), but the difference was not statistically significant (χ^2=3.48, P>0.05).

CONCLUSION: rCagA1 produced by the prokaryotic expression system constructed in this study possesses good immunoreactivity and antigenicity, and the established ELISAs can be used to detect CagA of *H pylori* and its antibody. *H pylori* isolates show high frequencies of *cagA* gene and CagA expression, but the infections by CagA$^+$ *H pylori* strains are not the most decisive factors to cause gastric diseases.

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INTRODUCTION

In China, gastritis and peptic ulcer are the most prevalent gastric diseases, and gastric cancer remains one of the most devastating malignant tumors with the highest morbidity[1-20]. *Helicobacter pylori* (*H pylori*) has been recognized as a human-specific gastric pathogen that colonizes in the stomach of at least half of the world's populations[21, 22]. Most infected individuals are asymptomatic, whereas in some cases, the infection causes acute, chronic gastritis or peptic ulceration, and plays an important role in the development of peptic ulcer and gastric adenocarcinoma, mucosa-associated lymphoid tissue (MALT) lymphoma and primary gastric non-Hodgkin's lymphoma[23-28].

So far, no evidence for the toxicity of the protein (CagA) expressed by cytotoxin-associated gene A (*cagA*) of *H pylori* has been presented[29,30]. However, previous studies demonstrated that CagA was closely associated with the pathogenicity of *H pylori* and severity of *H pylori*-related diseases[29-32]. Many epidemiological data indicated that the positive rate of *cagA* gene was significantly higher in the *H pylori* strains isolated from patients with peptic ulcer than in those with gastritis[33]. Patients infected with *cagA*$^+$ *H pylori* had a higher risk of developing gastric cancers than those infected with *cagA*$^-$ strains[34,35]. Approximately 60% to 70% of *H pylori* strains isolated from European and North American populations carried *cagA* gene[36-38], whereas over 90% of the isolates from Asia-Pacific populations were *cagA* gene-positive[39-42]. Strong antigenicity of CagA usually induces antibody in patients with *cagA*$^+$ *H pylori* infection and this antibody has been considered as a possible specific clinical indicator of *H pylori* infection[43-45]. However, the data are scarce concerning the correlations between the presence of *cagA*, CagA expression and antibody production, CagA$^+$ *H pylori* infection and types

of the resultant gastric diseases.

In the present study, a recombinant expression plasmid containing a relatively conserved *H pylori cagA* gene fragment 2 148 bp in length (*cagA*1) was constructed. *H pylori* strains in gastric biopsy specimens from patients with gastritis or peptic ulcer were isolated. The frequencies of *cagA* gene and expressions of *H pylori* isolates and CagA antibody in patients' sera were investigated. Furthermore, the correlations among $CagA^+$ *H pylori* infection and types of the resulted gastric diseases were also analyzed for the purpose of understanding the pathogenic effect of CagA and the potential of CagA antibody detection in clinical diagnosis of *H pylori* infection.

MATERIALS AND METHODS

Materials

A typical *H pylori* strain named Y06 isolated clinically was used to amplify *cagA*1 fragment. Primers for PCR amplification were synthesized by BioAsia (Shanghai, China). *Taq*-plus high fidelity PCR kit and restriction endonucleases were purchased from TaKaRa (Dalian, China). T-A cloning kit and sequencing service were provided by BBST (Shanghai, China). Plasmid *pET32a* as the expression vector and *E.coli* BL21DE3 as the host cell were purchased from Novagen (Novagen, Madison, USA). Rabbit antiserum against the whole cell of *H pylori*, HRP-labeling sheep antisera against rabbit IgG and human IgG were purchased from DAKO (Glostrup, Denmark) and Jackson ImmunoResearch (West Grove, USA), respectively. Agents used for isolation and identification of *H pylori* were purchased from BioMérieux (Marcy I' Etoile, France).

Gastric biopsy specimens with positive urease for *H pylori* isolation and serum samples for CagA antibody detection were collected from 156 patients in 4 hospitals in Hangzhou during Nov. 2001 to Feb. 2003. None of the patients had received nonsteroidal anti-inflammatory drugs, antacids or antibiotics within two weeks prior to the study. From the 156 patients, 126 biopsy specimens of *H pylori* isolates were obtained and 109 of the isolates survived after -70 ℃ storage. In the 109 patients (including 76 males and 33 females, with a mean age of 40.2 years), 61 patients suffered from chronic gastritis (41 chronic superficial gastritis, 10 chronic active gastritis, 10 chronic atrophy gastritis), and 48 had gastroduodenal ulcer (12 gastric ulcer, 30 duodenal ulcer, 6 gastric and duodenal ulcer).

Methods

Isolation and identification of *H pylori* Each of the biopsy specimen was homogenized with a tissue grinder and then inoculated on Columbia agar plates supplemented with 80 mL/L sheep blood, 5 g/L cyclodextrin, 5 mg/L trimethoprim, 10 mg/L vancomycin, 2.5 mg/L amphotericin B and 2 500 U/L cefsoludin. The plates were incubated at 37 ℃ under microaerobic conditions (50 mL/L O_2, 100mL/L CO_2 and 850 mL/L N_2) for 3 to 5 d. A bacterial isolate was identified as *H pylori* according to typical Gram staining morphology, positive results of biochemical tests for urease and oxidase, and slide agglutination with the commercial rabbit antibody against whole cell of the bacterium.

Preparation of DNA template Genomic DNA from each of the *H pylori* strains was extracted by conventional phenol-chloroform method and DNase-free RNase treatment. Concentration and purity of the DNA preparations were determined by ultraviolet spectrophotometry[46].

Polymerase Chain reaction The primers were designed to amplify *cagA*1 fragment from *H pylori* strain Y06 *cagA* gene based on the published data in GenBank. The sequence of sense primer with an endonuclease site of *Eco*R V was 5' -GCCGAT ATCATGATCAATAATCTTCAAGTAGC-3' , and that of the antisense primer with an endonuclease site of *Xho*I was 5' -CGGCTCGAGTTATGAAAT CCATTCTGGATTG-3' . The total volume per PCR was 100 μL, containing 2.5 mol/L each of the dNTP, 250 nmol/L each of the two primers, 15 mol/L $MgCl_2$, 3.0 U *Taq*-plus polymerase, 100 ng DNA template and 1×PCR buffer (pH 8.3). The parameters for PCR were at 94 ℃ for 5 min, ×1; at 94 ℃ for 30 s, at 50 ℃ for 30 s, at 72 ℃ for 120 s, ×10; at 94 ℃ for 30 s, at 50 ℃ for 30 s, at 72 ℃ for 130 s (additional 10 s for each of the following cycles), ×20; at 72 ℃ for 10 min, ×1.

To increase the positive detection rate of *cagA* gene, two sets of primers derived from different regions of *cagA* gene were applied in PCR. The sequences of F1/B1 primers were 5' -GATAACAGGCAAGCTTTTGAGG-3' (sense), 5' -CTGCAAAAGATTGTTTGGCAGA-3' (antisense)[31]. The sequences of D008/R008 primers were 5' -ATAATGCTAAAT TAGACAACTTGAGCG-3' (sense), 5' -TTAGAATAATCAA CAAACATCACGCCA-3' (antisense)[36]. Except for the primers and DNA templates, all the other reagents and reaction volumes used in PCR for *cagA* detection were the same as in *cagA*1 amplification. The parameters for the two PCRs were at 94 ℃ for 5 min, ×1; at 94 ℃ for 30 s, at 55 ℃ for 1 min, at 72 ℃ for 90 s, ×35; at 72 ℃ for 7 min, ×1.

The results of PCR were observed under UV light after electrophoresis on 1.5% agarose gel pre-stained with ethidium bromide. The expected sizes of *cagA*1 amplification fragment and the two target amplification fragments for *cagA* gene detection were 2 172 bp (including ATG, TAA, and a 18-bp sequence containing endonuclease sites and protective nucleotide residuals), 349 bp and 298 bp, respectively.

Cloning and sequencing The *cagA*1 amplification fragment was cloned into plasmid vector *pUCm-T* (*pUCm-T-cagA*1) by using the T-A cloning kit according to the manufacturer' s instructions. The recombinant plasmid was amplified in *E.coli* DH5α and then extracted by Sambrook' s method[46]. A professional company (BBST) was responsible for nucleotide sequence analysis of the inserted fragment. Two plasmids *pUCm-T-cagA*1 and *pET32a* were extracted from two different strains of *E.coli* DH5α after amplification in LB medium and then digested with *Eco*RV and *Xho*I, respectively[46]. The fragments *cagA*1 and *pET32a* were recovered and ligased. The recombinant expression vector *pET32a-cagA*1 was transformed into *E.coli* BL21DE3, and the expression system designated as *pET32a-cagA*1-*E.coli* BL21DE3. The *cagA*1 fragment inserted in *pET32a* was sequenced again.

Expression and identification of target recombinant protein *pET32a-cagA*1-*E.coli* BL21DE3 was rotatively cultured in LB medium at 37 ℃ under induction with isopropylthio-β-D-galactoside (IPTG) at different concentrations of 1.0, 0.5 and 0.1 mmol/L, respectively. The supernatant and precipitate of the culture after incubation were separated by centrifugation and then the bacterial pallets were ultrasonically fragmented (300 V, 5 s×3). SDS-PAGE was used to measure the molecular mass and output of the target recombinant protein (rCagA1). Ni-NTA affinity chromatography was applied to collect rCagA1. The commercial rabbit antiserum against whole-cell *H pylori* and HRP-labeling sheep antiserum against rabbit IgG were used as the first and second antibodies to determine the immunoreactivity of rCagA1 by Western blotting, respectively. Rabbits were immunized with rCagA1 to prepare antisera. Immunodiffusion assay was performed to determine the antigenicity of rCagA1.

Enzyme-linked immunosorbent assay (ELISA) By using rCagA1 as the coating antigen at the concentration of 20 μg/mL, with the serum sample (1:400 dilution) from a patient as the first antibody and HRP-labeling sheep antibody against human IgG (1:4 000 dilution) as the second antibody, CagA antibody in the sera of the 126 *H pylori*-infected patients was detected. The result of ELISA for a patient' s serum sample was

considered positive if the value of optical density at 490 nm (OD_{490}) exceeded the mean plus 3 standard deviations of 6 different negative serum samples[47]. CagA expression in *H pylori* isolates was examined using ultrasonic supernatant of each of the *H pylori* isolates (50 μg/mL) as the coating antigen, self-prepared rabbit anti-rCagA1 serum (1:800 dilution) as the first antibody and HRP-labeling sheep antibody against rabbit IgG (1:3 000 dilution) as the second antibody. The result of ELISA for a *H pylori* ultrasonic supernatant sample was considered positive if its OD_{490} value was over the mean plus 3 standard deviations of 6 separated *E.coli* DH5α ultrasonic supernatant samples at the same protein concentration[47].

Analysis of correlation among *cagA* gene, CagA and its antibody and *H pylori*-related diseases According to the clinical data and the obtained results, the correlations among infection with *H pylori* carrying *cagA* gene and expressing CagA isolated from the patients' gastric biopsy specimens, and the type and severity of gastric diseases in the same patient were analyzed.

Statistical analysis

The nucleotide and putative amino acid sequences of the cloned *cagA*1 fragment were compared for homologies with the published corresponding sequences (GenBank accession No. AB015416). χ^2 test was applied to analyze the clinical data, PCR results for *cagA* detection and ELISA results for CagA detection.

RESULTS

Positivity rate of H pylori in clinical isolates

In the 156 gastric biopsy specimens with positive urease, *H pylori* was detectable in 126 specimens, with a positivity rate of 80.8%.

PCR results

Using the primer pairs F1/B1 and D008/R008 respectively, 82.6% and 78.9% of the tested *H pylori* isolates (90/109) were positive for *cagA* gene, and the total *cagA* gene positivity rate was 97.2%(106/109). The target amplification products of *cagA*1 from *H pylori* strain Y06 and two fragments for *cagA* gene detection from the isolates are shown in Figure 1.

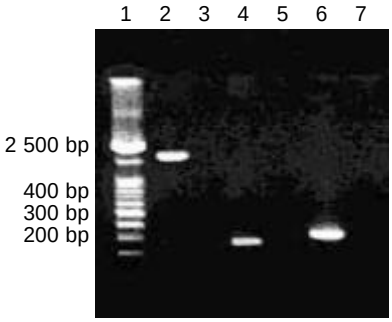

Figure 1 Target amplification fragments of *cagA gene* amplified from *H pylori* isolates with different primers. Lane 1: 100 bp DNA marker; Lanes 2, 4 and 6: Target amplification fragments by using *cagA*1, F1/B1 and D008/R008 primers, respectively; Lanes 3, 5 and 7: Blank controls.

Nucleotide sequence analysis

The nucleotide sequences of *cagA*1 fragment in *pUCm-T-cagA*1 and *pET32a-cagA1* were completely the same. The nucleotide and putative amino acid sequences of the cloned *cagA*1 fragment showed 94.83% (Figure 2) and 93.30% (Figure 3) homologies with the published sequences from *H pylori* strain NCTC11637 (GenBank accession No.: AB015416), respectively.

```
(1) 61  ///TTTATCAATAATCTTCAAGTAGCTTTTCTCAAAGTTGATAACGCTGTCGCTTCATAC
(2) 61    ATG.....T.....

(1)121  GATCCTGATCAAAAATCAATCGTTGATAAGAACGATAGGGATAACAGGCAAGCTTTTGAA
(2)121  .....C.....T.....T

(1)181  GGAATCTCGCAATTAAGGGAAGAATACTCCAATAAAGCGATCAAAAATCCTACCAAAAAG
(2)181  .....

(1)241  AATCAATATTTTTCAGACTTTATCAATAAGAGCAATGATTTAATCAACAAAGACAATCTC
(2)241  .....G.....

(1)301  ATTGATGTAGATTCTTCCACAAAGAGCTTTCAGAAATTTGGGGATCAGCGTTACCGAATT
(2)301  ......A...G.....T..GA.....AC.....

(1)361  TTCACAAGTTGGGTGTCCCATCAAAACGATCCGTCTAAAATCAACACCCGATCGATCCGA
(2)361  .....

(1)421  AATTTTATGGAAAATATCATACAACCCCCTATCCCTGATGATAAAGAGAAAGCGGAGTTT
(2)421  .....C.....A.....A.....

(1)481  TTGAAATCTGCCAAACAATCTTTTGCAGGAATCATTATAGGGAATCAAATCCGAACGGAT
(2)481  .....

(1)541  CAAAAATTCATGGGCGTGTTTGATGAGTCCTTGAAAGAAAGGCAAGAAGCAGAAAAAAAT
(2)541  .....G.....A..T.....

(1)601  GGAGAGCCTACTGGTGGGGATTGGTTGGATATTTTTCTCTCATTTATATTTGACAAAAAA
(2)601  .....T.A.....G.....A.....G..

(1)661  CAATCTTTCGATGTCAAAGAAACAATCAATCAAGAACCAGTTCCCCATGTCCAACCAGAT
(2)661  .....CT.....G.....

(1)721  TTAGCCACTACCACCACCGACATACAAGGCTTACCGCCTGAAGCTAGAGATTTACTTGAT
(2)721  A.....C.....T....G.....G.....

(1)781  GAAAGGGGTAATTTTTCTAAATTCACTCTTGGCGATATGGAAATGTTAGATGTTGAGGGA
(2)781  .....C
```

(1)841 GTCGCTGACATTGATCCCAATTACAAGTTCAATCAATTATTGATTCACAATAACGCTCTG
(2)841 .....C.....T.....

(1)901 TCTTCTGTGTTAATGGGGAGTCATAATGGCATAGAACCTGAAAAAGTTTCATTGTTGTAT
(2)901 .....A.....

(1)961 GGGGGCAATGGTGGTCCTGGAGCTAGGCATGATTGGAACGCCACCGTTGGTTATAAAGAC
(2)961 .C.....TT.....C.A...C.....A..

(1)1021 CAACAAGGCAGCAATGTGGCTACAATAATTAATGTGCATATGAAAAACGGCAGTGGCTTA
(2)1021 .....GA.....C.C.....

(1)1081 GTCATAGCAGGTGGTGAGAAAGGGATTAACAACCCTAGCTTTTATCTCTATAAAGAAGAC
(2)1081 .....T.....C.....

(1)1141 CAACTCACAGGCTCACAACGAGCATTAAGTCAAGAAGAGATCCAAAACAAAATAGATTTC
(2)1141 .....G.....G.....

(1)1201 ATGGAATTTCTTGCACAAAATAATGCTAAATTAGACAACTTGAGCAAGAAAGAGAAGGAA
(2)1201 .....C.....G.....A...

(1)1261 AAATTCCGAACTGAGATTAAAGATTTCCAAAAAGACTCTAAGGCTTATTTAGACGCCCTA
(2)1261 .....A..A.....G.....T.....

(1)1321 GGGAATGATCGTATTGCTTTTGTTTCTAAAAAAGACACAAAACATTCAGCTTTAATTACT
(2)1321 .....C.....C.....

(1)1381 GAGTTTGGTAATGGGGATTTGAGCTACACTCTCAAAGATTATGGGAAAAAAGCAGATAAA
(2)1381 .....G.....G.

(1)1441 GCTTTAGATAGGGAGAAAAATGTTACTCTTCAAGGTAGCCTAAAACATGATGGCGTGATG
(2)1441 .....G.....A.....A.....

(1)1501 TTTGTCAATTATTCCAATTTCAAATACACCAACGCTTCCAAGAGTCCTGATAAGGGCGTG
(2)1501 .....T.....T.....C.....T...

(1)1561 GGCGTTACGAATGGCGTTTCGCATTTAGAAGTAGGCTTTAACAAGGTAGCTATCTTTAAT
(2)1561 .....A.....C.....C.C.....G.....G.....

(1)1621 TTGCCTGATTTAAATAATCTCGCTATCACTAGTTACGTAAGGCGGAATTTAGAGGATAAA
(2)1621 .....T.....A.....

(1)1681 CTAACCACTAAAGGATTGTCCCCACAAGAAGCTAATAAGCTTATCAAAGATTTTTTGAGC
(2)1681 ...GT....G.....T.....

(1)1741 AGCAACAAAGAATTGGTTGGAAAAACTTTAAACTTCAATAAAGCTGTAGCTGACGCTAAA
(2)1741 .....G.....T.....

(1)1801 AACACAGGCAATTATGATGAAGTGAAAAAAGCTCAGAAAGATCTTGAAAAATCTCTAAGG
(2)1801 .....C.....

(1)1861 AAACGAGAGCATTTAGAGAAAGAAGTAGAGAAAAAATTGGAGAGCAAAAGCGGAAACAAA
(2)1861 .....C.....

(1)1921 AATAAAATGGAAGCAAAAGCTCAAGCTAACAGCCAAAAAGATGAGATTTTTGCGTTGATC
(2)1921 .....G.....A.....

(1)1981 AATAAAGAGGCTAATAGAGACGCAAGAGCAATCGCTTACGCTCAGAATCTTAAAGGCATC
(2)1981 .....G.....T.....

(1)2041 AAAAGGGAATTGTCTGATAAACTTGAAAATGTCAACAAGAATTTGAAAGACTTTGATAAA
(2)2041 .....AA.....G.....AG....

(1)2101 TCTTTTGATGAATTCAAAAATGGCAAAAATAAGGATTTCAGCAAGGCAGAAGAAACACTA
(2)2101 .....T.....G...

(1)2161 AAAGCCCTTAAAGGTTCGGTGAAAGATTTAGGTATCAATCCAGAATGGATTTCA
(2)2161 .....C.....A.....TAA

Figure 2 Comparison of nucleotide sequence homology of *cagA*1 fragments between different *H pylori* strains. (1): Corresponding nucleotide sequence of *cagA*1 fragment from *H pylori* strain NCTC11637. (2): Sequencing result of *cagA*1 fragment from *H pylori* strain Y06. "/ / /" indicates the deletion mutation of the nucleotide residuals. Underlined is the position of the primers.

(1) 1 INNLQVAFLKVDNAVASYDPDQKSIVDKNDRDNRQAFEGISQLREEYSNKAIKNPTKKNQ
(2) 1 .....P.....D.....

(1) 61 YFSDFINKSNDLINKDNLIDVESSTKSFQKFGDQRYRIFTSWVSHQNDPSKINTRSIRNF
(2) 61 .....IG...R.....T.....

(1) 121 MENIIQPPILDDKEKAEFLKSAKQSFAGIIIGNQIRTDQKFMGVFDESLKERQEAEKNGE
(2) 121 .....P.....F.....

(1) 181 PTGGDWLDIFLSFIFDKKQSSDVKEAINQEPVPHVQPDIATTTTDIQGLPPEARDLLDER
(2) 181 .....V.N.E.....H.....S.....

(1) 241 GNFSKFTLGDMEMLDVEGVADIDPNYKFNQLLIHNNALSSVLMGSHNGIEPEKVSLLYGG
(2) 241 .....D.....A.

(1) 301 NGGPGARHDWNATVGYKDQQGNNVATIINVHMKNGSGLVIAGGEKGINNPSFYLYKEDQL
(2) 301 ....F..K.....N...D....L.....

(1) 361 TGSQRALSQEEIQNKIDFMEFLAQNNAKLDNLSEKEKEKFRTEIKDFQKDSKAYLDALGN
(2) 361 .....R.....QN..E.....

(1) 421 DRIAFVSKKDTKHSALITEFGNGDLSYTLKDYGKKADKALDREKNVTLQGSLKHDGVMFV
(2) 421 .....P.....K.....R.....E.....N.....

(1) 481 DYSNFKYTNASKNPNKGVGVTNGVSHLEVGFNKVAIFNLPDLNNLAITSFVRRNLEDKLT
(2) 481 N.....S.D.....DA..S...V.....N..V

(1) 541 TKGLSPQEANKLIKDFLSSNKELVGKTLNFNKAVADAKNTGNYDEVKKAQKDLEKSLRKR
(2) 541 .E...L.....A.....

(1) 601 EHLEKEVEKKLESKSGNKNKMEAKAQANSQKDEIFALINKEANRDARAIAYAQNLKGIKR
(2) 601 .....K.....S.....

(1) 661 ELSDKLENVNKNLKDFDKSFDEFKNGKNKDFSKAEETLKALKGSVKDLGINPEWIS
(2) 661 .....KI..D....S.....

Figure 3 Comparison of putative amino acid sequence homology of *cagA*1 fragment between different *H pylori* strains. (1): Corresponding putative amino acid sequence of *cagA*1 fragment from *H pylori* strain NCTC11637. (2): Putative amino acid sequence of *cagA*1 fragment from *H pylori* strain Y06. Underlined is the position of the primers.

Expression of target recombinant protein

IPTG at concentrations of 1.0, 0.5 and 0.1 mmol/L could efficiently induce the expression of rCagA1, which was detected mainly in the ultrasonic precipitate with an output of approximately 30% of the total bacterial proteins (Figure 4).

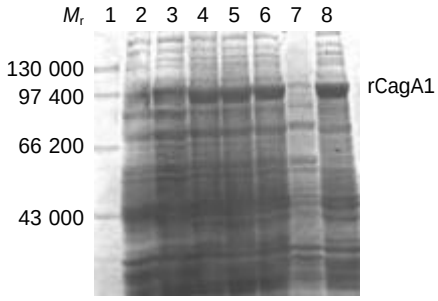

Figure 4 rCagA1 expression by *pET32a-cagA*1-*E.coli* BL21DE3 induced with IPTG. Lane 1: Protein marker; Lane 2: Blank control; Lane 3: Non-induced; Lanes 4-6: induced with 0.1, 0.5 and 1.0 mmol/L IPTG, respectively; Lanes 7 and 8: Bacterial supernatant and precipitate induced with 0.5 mmol/L IPTG, respectively.

Immunoreactivity and antigenicity of rCagA1

The commercial rabbit antibody against whole-cell *H pylori* could bind to rCagA1 as confirmed by Western blotting (Figure 5). Immunodiffusion assay demonstrated a titer of 1:4 between rCagA1 and rabbit anti-rCagA1 serum.

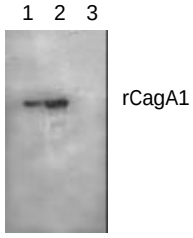

Figure 5 Western blotting of binding between rCagA1 and commercial rabbit antiserum against whole-cell *H pylori.* Lanes 1 and 2: 20 μL and 40 μL of rCagA1 extract, respectively; Lane 3: Blank control.

ELISA results

The mean A_{490} values (mean±SD) of the 6 negative serum samples was 0.37±0.03 in the detection of specific antibodies in sera of patients, and the positive reference value of 0.46 was consequently derived. According to the reference value, 88.1% (96/109) of the tested patients' serum samples were positive for the rCagA1 antibodies with an A_{490} value ranging from 0. 56 to 1.05 (Table 1). From the mean A_{490} values of the 5 negative bacterial controls (0.27±0.09) in the detection of CagA1 in *H pylori* isolates, the positive reference value of 0.54 was

derived. According to the reference value, the epitope of rCagA1 with an A_{490} value ranging from 0.55% to 0.9792.6%(101/109) was detected in the tested *H pylori* isolates (Table 1).

Table 1 Detection of CagA expression in *H pylori* isolates and CagA antibody in infected patients' sera

Tested indicator	Tested cases	Positive cases	Negative cases	Positivity rate (%)
CagA gene	109	106	3	97.2
CagA protein	109	101	8	92.6
Anti-CagA	109	96	13	88.1

Correlation between CagA and gastric diseases

A high rate of 97.9% of *H pylori* isolated from the peptic ulcer specimens (47/48) and 88.5% from the gastritis specimens (54/61) was positive for CagA expression, showing no statistically significant difference between the two positive rates (χ^2=3.48, P>0.05). Of the 8 *H pylori* strains in which CagA expression failed to be detected, 1, 1 and 6 strains were isolated from the biopsy specimens of gastric ulcer, chronic active gastritis and chronic superficial gastritis, respectively, and this distribution did not show any statistically significant difference, either (χ^2=1.23, P>0.05).

DISCUSSION

CagA expressed by *H pylori* was demonstrated to induce cellular skeleton rearrangement and interleukin (IL)-8 secretion in gastric epithelial cells[31,32]. IL-8, recognized as an inflammatory cytokine, could cause inflammation by inducing gathering of neutrophilic cells[48,49]. Infection with *cagA*$^+$ *H pylori* may elevate the risks of atrophic gastritis, intestinal metaplasia and gastric adenocarcinoma[34,50,51], and CagA is therefore considered as the most important pathogenic factor of *H pylori.*

CagA gene had a single copy located at the terminal end of region I in cag pathogenic island (CPI)[31,52], and was prone to mutation, especially in the 3' -end by insertion of different numbers of repeated sequences, resulting in the great variation in its length ranging from 3 444 to 5 925 bp in different isolates[36,37]. According to the analysis of 37 *cagA* gene sequences from GenBank, a fragment with approximate 65 bp starting from the 5' -end of *cagA* gene of different *H pylori* isolates also exhibited frequent mutations such as replacement, insertion and deletion, *etc*. Therefore, a relatively conserved fragment of 2 148 bp from the 67th to 2 214th bp at 5' -end of *cagA* gene was selected for cloning, which was provisionally named as *cagA*1. In this study, homologies of the nucleotide and amino acid sequences of the cloned *cagA*1 fragment reached 94.83% and 93.30% respectively in comparison with the reported sequences in GenBank (accession No.AB015416). High output of rCagA1, approximately 30% of the total bacterial protein, expressed by the constructed recombinant prokaryotic expression system *pET32a-cagA*1-*E.coli* BL21DE3 was confirmed by SDS-PAGE. rCagA1 could be recognized by a commercial antibody against whole-cell *H pylori* and was able to induce rabbit to produce high-titer antibodies, indicating that rCagA1 with good immunoreactivity and antigenicity can be used in ELISA as a qualified antigen for detecting CagA antibody and for preparing animal antiserum to detect CagA.

Yang *et al* reported that all the *cagA*$^+$ *H pylori* isolates were capable of expressing CagA[41]. However, we found that 5 strains of *cagA*$^+$ *H pylori* (7.3%) failed to exhibit the expression. Considering the highly likely mutation in *cagA* genes from different *H pylori* isolates, this non-expression of CagA was probably due to sequence mutation or abnormal transcription and translation[36,37]. In the present study, 88.1% of the serum samples from the *H pylori*-infected patients (96/109) were positive for CagA antibody, and the positive rate was only slightly lower than those of *cagA* gene (97.2%) and CagA (92.6%) of the isolates, suggesting that CagA possesses strong antigenicity and usually induces detectable specific antibody in *cagA*$^+$ *H pylori*-infected patients. However, we found in this study that 11.9% of the serum samples were negative for CagA antibody (13/109), including 3 cases (2.8%) of *cagA*$^-$ *H pylori* infection, 5(4.6%) of *cagA*$^+$ *H pylori* infection and 5(4.6%) of CagA$^+$ *H pylori* infection. In addition, previously published data and our results suggest that over 90% of the *H pylori* isolates from Asia-Pacific areas were *cagA* gene-positive[39-42], whereas 60% to 70% of the *H pylori* isolates from European and North American areas were positive[36-38], indicating that the presence of CagA antibody could be used as a reference indicator with only a small risk of error for detecting *H pylori* infection in individuals from Asia-Pacific areas, but not for those from European or North American areas. It should be noted that the positive rate of *cagA* gene in the *H pylori* isolates in this study was as low as 78.9% to 82.6%, as detected using a single pair of primers F1/B1 or D008/R008, indicating that using multiple pairs of primers in PCR may increase the positive rate for *cagA* gene detection.

Covacci *et al* and Figueiredo *et al* reported that *cagA*$^+$ *H pylori* infection could usually cause serious gastric diseases[37,38]. For example, 90% of *H pylori* isolates from peptic ulcer patients were *cagA* gene-positive, while only 50% to 60% of the isolates from superficial gastritis were positive. However, the reports from Asia-Pacific areas did not show a definite correlation between *cagA*$^+$ *H pylori* infection and severity of the diseases[39-42]. Although *cagA*$^+$ *H pylori* was isolated from chronic gastritis patients at a higher rate (97.9%) than from peptic ulcer patients (88.5%), the difference was not statistically significant (χ^2=3.48, P>0.05), probably due to the high rate of *cagA* gene-carrying *H pylori* (97.2%) and a relative small population tested in this study.

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